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MICRORNA BIOMARKERS FOR PERIPHERAL BLOOD FRACTIONS IDENTIFICATION: possible forensic applications

Dissertação de Candidatura ao grau de Mestre em Medicina Legal submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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INFORMAÇÃO TÉCNICA

TÍTULO:
microRNA biomarkers for peripheral blood fractions identification: possible forensic applications

Dissertação de Candidatura ao Grau de Mestre em Medicina Legal, apresentada ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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“Don't be afraid of hard work. Nothing worthwhile comes easily.” – Gertrude B. Elion

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ABBREVIATIONS

A

ACC	Accuracy
AGO	Argonaute
AKE	Ammonium chloride, potassium hydrogen phosphate and EDTA
AUC	Area Under the Curve

C

cDNA	Complementary Deoxyribonucleic Acid
Ct	Cycle threshold

D

DNA	Deoxyribonucleic acid

E

EDTA	Ethylenediaminetetraacetic Acid

H

HDL	High Density Lipoprotein

M

miR	microRNA
miRNA	microRNA
mL	milliliter
mRNA	Messenger Ribonucleic Acid
MVs	Microvesicles

N

NPV	Negative Predictive Value

O

OR	Odds ratio

P

PBS	Phosphate Buffered Saline
pg	picogram
PPV	Positive Predictive Value or Precision
pre-miRNA	miRNA precursor
pri-miRNA	Primitive miRNA
PTH	Parathyroid Hormone

Q

qPCR	Real-time polymerase chain reaction

R

RBCs	Red Blood Cells
RCC	Renal Cell Carcinoma
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNase	Ribonuclease
ROC	Receiver Operating Characteristic
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction

U

μL	microliter

V

vs.	*versus*

W

WBCs	White Blood Cells

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Abstract

ABSTRACT

Introduction: MicroRNAs (miRNAs) are small non-coding RNAs, with a length of 18 to 24 nucleotides that play a regulative role in several cellular processes. Since their discovery, they have been identified in organs, tissues, cells and body fluids and their potential as molecular biomarkers for the diagnosis of various pathologic conditions, as well as for forensic body fluid identification has been explored. In the particular case of peripheral blood, this body fluid is normally seen as a whole despite the fact that there are several differences among its different fractions (plasma, serum and blood cells). One of these differences relies in the miRNA population, since that some miRNAs show different expression levels and patterns according to the fraction analyzed. Therefore, in order to standardize the results of studies involving miRNAs, and eventually the analysis of forensic evidences, it is extremely important to assess the miRNA expression pattern among the different fractions of peripheral blood and define a specific profile.

Objectives: In this study we evaluated the miRNA expression pattern of miR-801, miR-369-3p, miR-16-5p and miR-24-1-5p in the fractions of peripheral blood (plasma, serum, and white blood cells (WBCs)), in order to identify specific miRNAs of these fractions.

Material & Methods: The peripheral blood samples were processed and the miRNAs were extracted from the serum, plasma and WBCs samples. Complementary DNA (cDNA) synthesis and quantitative real-time PCR were then performed, followed by a statistical analysis of the obtained results with the software *IBM®SPSS®Statistics* (Version 22.0).

Results & Discussion: We found that the analyzed miRNAs were differently expressed across the plasma, serum and WBCs samples tested. Our results indicated that miR-24-1-5p was detected almost exclusively in WBCs and had a specificity of 81.82%, and a sensitivity of 90.91% for the identification of WBCs *versus* serum and plasma. Furthermore, miR-801, miR-369-3p and miR-16-5p exhibited the higher expression levels in serum, leading us to conclude that this is the optimal fraction for miRNA analysis. Additionally, as these three miRNAs could be found across all peripheral blood fractions tested, they could be biomarkers for whole blood identification. MiR-16-5p was found to be significantly more expressed in serum, in comparison to plasma and WBCs, however, in its use as a serum biomarker, one should take into account the influence of gender. We also found that, overall, the expression levels of the tested miRNAs didn't exhibited any major differences between samples from individuals of different gender or age, what is an important factor in the development of biomarkers for forensic procedures, since a good biomarker should remain unaltered throughout the population, in order to allow the correct identification of the peripheral blood fraction regardless of individual characteristics.

Furthermore, for miR-24-1-5p, the odds ratio (OR), specificity, sensitivity, positive predictive value or precision (PPV), negative predictive value (NPV) and accuracy (ACC) values remained elevated regardless of gender and age sub-divisions.

Conclusion: Our results indicate that miR-801, miR-369-3p and miR-16-5p have the potential to be biomarkers for whole peripheral blood identification. However, before its implementation in forensic analysis, further studies with other body fluids are needed, in order to confirm whether or not they can be found in other body fluids. Moreover, miR-16-5p could also be a biomarker for serum identification and miR-24-1-5p for WBCs identification.

Resumo

RESUMO

Introdução: Os microRNAs (miRNAs) são pequenas moléculas de RNA não codificante, com um comprimento de 18 a 24 nucleótidos, que desempenham um papel regulador em diversos processos celulares. Desde a sua descoberta, os miRNAs têm sido detetados em órgãos, tecidos, células e fluidos corporais, sendo que o seu potencial como biomarcadores moleculares para o diagnóstico de várias condições patológicas, assim como para a identificação forense de fluidos corporais tem sido explorado. No caso particular do sangue periférico, este fluido corporal é normalmente visto como um todo, apesar do facto de apresentar várias diferenças entre as suas frações (plasma, soro e células sanguíneas). Uma destas diferenças consiste no padrão de expressão de miRNAs, uma vez que alguns miRNAs apresentam diferentes níveis e padrões de expressão de acordo com a fração analisada. Desta forma, torna-se relevante o estabelecimento do perfil de miRNAs específico associado às diferentes frações de sangue periférico, o que poderá ser muito útil em Medicina Legal, nomeadamente na análise de provas forenses.

Objetivos: Neste estudo avaliou-se o padrão de expressão dos miRNAs miR-801, miR-369-3p, miR-16-5p e miR-24-1-5p, nas diferentes frações de sangue periférico (plasma, soro e leucócitos), de modo a identificar miRNAs específicos destas frações.

Material & Métodos: As amostras de sangue periférico foram processadas e os miRNAs foram extraídos a partir das amostras de soro, plasma e leucócitos. Após a síntese de DNA complementar, os miRNAs foram analisados por PCR quantitativo em tempo real e os resultados obtidos foram analisados estatisticamente com recurso ao software estatístico *IBM®SPSS®Statistics* (Versão 22.0).

Resultados & Discussão: Verificou-se que os miRNAs analisados se encontravam diferencialmente expressos nas amostras testadas de plasma, soro e leucócitos. Os resultados indicaram que o miR-24-1-5p foi detetado quase exclusivamente em leucócitos, apresentando uma especificidade de 81,82% e uma sensibilidade de 90.91% para a identificação de leucócitos *versus* soro e plasma. Adicionalmente, o miR-801, o miR-369-3p e o miR-16-5p exibiram níveis de expressão mais elevados no soro, o que leva a concluir que esta é a fração ideal para a análise de miRNAs. Atendendo que estes três miRNAs podem ser encontrados em todas as frações de sangue periférico testadas, podemos considerar que estes poderão ser potenciais biomarcadores para a identificação de sangue total. O miR-16-5p apresentou uma expressão significativamente mais elevada no soro, em comparação com o plasma e os leucócitos, no entanto, a sua utilização como um biomarcador de soro deve ser feita com precaução atendendo à influência do género encontrada no presente estudo. Observamos também que, de um modo geral, os níveis

de expressão dos miRNAs testados não exibiram diferenças significativas entre amostras de indivíduos de diferente género ou idade, o que é um fator importante no desenvolvimento de biomarcadores para procedimentos forenses, uma vez que um bom biomarcador deve manter-se inalterado em toda a população, de modo a permitir uma correta identificação da fração de sangue periférico. Quanto ao miR-24-1-5p, verificamos que os valores de razão de possibilidades, especificidade, sensibilidade, valor preditivo positivo ou precisão, valor preditivo negativo e acurácia permaneceram elevados independentemente das sub-divisões de género e idade.

Conclusão: O miR-801, o miR-369-3p e o miR-16-5p apresentam um elevado potencial de serem biomarcadores moleculares úteis na identificação de sangue periférico. Porém, antes da sua implementação nas análises e procedimentos forenses, são necessários estudos adicionais que comparem outros fluidos corporais, de modo a confirmar se estes miRNAs podem ou não ser encontrados noutros fluidos. O presente estudo revelou ainda que o miR-16-5p poderá ser um biomarcador para a identificação de soro e o miR-24-1-5p para identificação de leucócitos.

1. Introduction

1. INTRODUCTION

The different fractions of peripheral blood (serum, plasma and blood cells), despite being normally seen as a whole, show several differences among them. This is the case of the protein fibrinogen, which can be found in plasma, but is absent in serum [1]. Other variation between the fractions is the microRNA (miRNA) expression levels within the different peripheral blood fractions. In the literature there are some studies that point to the differentiated expression of certain miRNAs in blood fractions, however given the lack of information in this area, a better characterization of the miRNA content is needed [2, 3]. Therefore, it is important to address this subject in order to, not only, establish and characterize the existing differences, but also to standardize the methodologies used. Thus, once the miRNA expression pattern in the peripheral blood fractions is established it will facilitate and allow the implementation of miRNA analysis in forensic procedures.

In fact, in comparison to other nucleic acids, such as the deoxyribonucleic acid (DNA) and the messenger ribonucleic acid (mRNA), miRNAs are smaller, what makes them more stable and less prone to degradation [4, 5]. Moreover, these molecules exhibit different expression profiles according to the tissues or body fluid tested [5]. Due to these characteristics, they have a great potential of being reliable and efficient biomarkers for body fluid characterization, as well as for the diagnosis of pathologic conditions.

1.1. microRNAs

MiRNAs are short non-coding RNA, with a length of 18 to 24 nucleotides that play a regulative role in several cellular processes. Currently, there are, approximately 1881 known sequences of human miRNAs (miRBase release 21.0) [6, 7].

MiRNAs are transcribed from the genomes of nucleated cells by RNA polymerase II or III and these primary miRNA transcripts (pri-miRNAs) are then modified by the addition of a 5’ cap and a 3’ poly-A tail [4]. Subsequently, the pri-miRNAs are processed first in the nucleus by the ribonuclease (RNase) III enzyme Drosha, being later exported by Exportin 5 to the cytosol, where they are processed by the RNase III enzyme Dicer [4, 8]. This processing of pri-miRNAs originates initially a miRNA precursor (pre-miRNA) with approximately 70 nucleotides of length, and finally a smaller and mature double-stranded miRNA of 18–24 nucleotides [4, 8]. One strand of this mature miRNA is incorporated in the RNA-induced silencing complex (RISC), where it can regulate the expression of target mRNAs [4]. This miRNA can induce post-transcriptional gene silencing, through the

guidance of the RISC complex to the target mRNAs, or mRNA cleavage and degradation (Figure 1) [8, 9]. The RISC complex is also responsible for the process RNA interference, in which a double-stranded RNA silences the expression of homologous genes by mRNA cleavage [8, 10-12]. The other strand could be degraded, or prepared for being exported from the cell [4]. Thus, miRNAs could be found not only on the intracellular compartments, but also in the extracellular microenvironment, namely in serum, plasma and urine [13]. However, there is little information concerning the subject of the origin of circulating miRNAs (both in healthy and sick individuals) and what factors may influence the levels of circulating miRNAs [14].

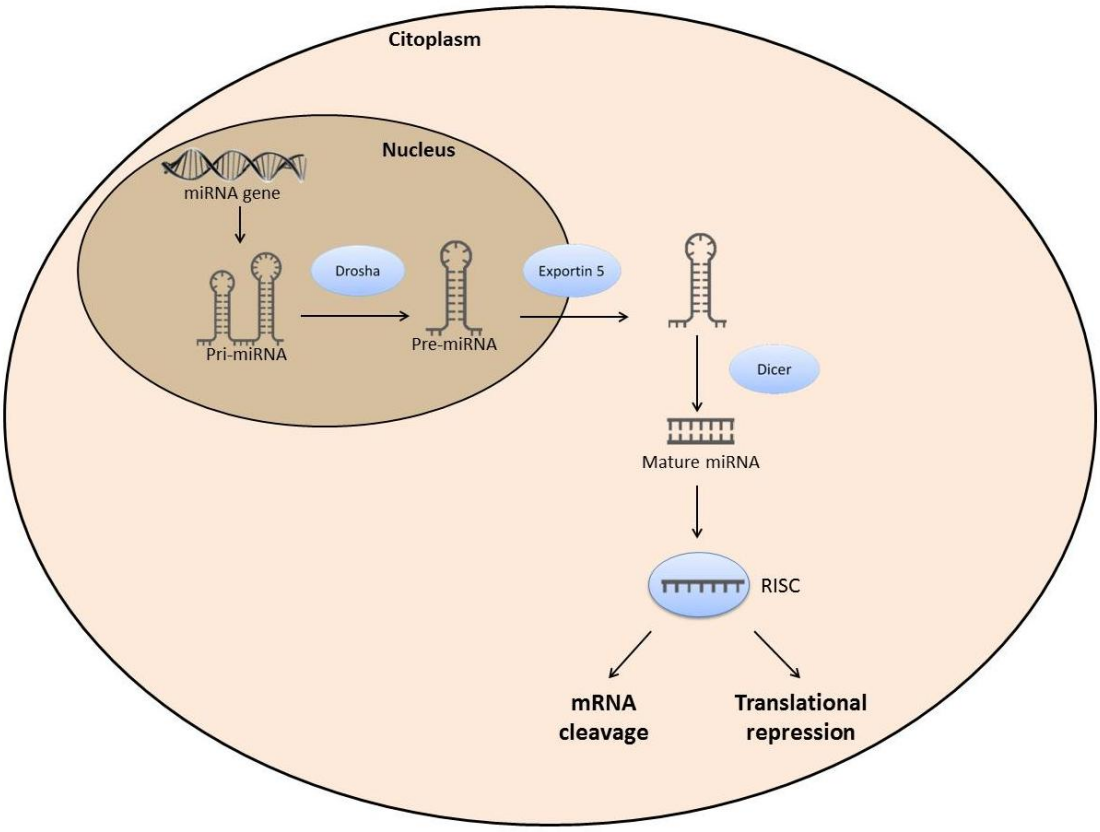

Figure 1 – Biogenesis and post-transcriptional suppression of microRNAs. MiRNAs are transcribed from the genomes of nucleated cells into pri-miRNA transcripts, which are processed by Drosha in the nucleus, originating ~70-nucleotide pre-miRNAs. These pre-miRNAs are transported to the cytoplasm by Exportin 5 and are processed by Dicer into a mature double-stranded miRNA of 18-24 nucleotides. One strand of this miRNA is incorporated into the RNA-induced silencing complex (RISC), which later can induce translational repression or mRNA cleavage.

Nevertheless, when compared to DNA and mRNA, the small size of miRNAs makes them more stable and, therefore, less prone to degradation, making them good options for the development of biomarkers. Another factor that plays a key role in their stability is the argonaute (AGO) proteins, which are a catalytic component of the RISC complex [10-12, 15]. The interaction of AGO proteins with mature miRNAs makes them much more stable to the degradation process [6, 11, 16]. In fact, Chen and colleagues demonstrated that serum miRNAs were able to remain stable even after being exposed to severe environmental conditions, like high temperatures, extreme pH and prolonged storage [17].

Another characteristic of miRNAs is the differential expression of these molecules among tissues and fluids [4, 5]. For example, the miR-124 is considered a brain-specific miRNA and miR-122 is highly expressed in liver [3, 4, 18, 19]. Alterations on the normal miRNA expression pattern have also been associated with a diversity of pathologic conditions, such as cancer or cardiac diseases [19-28].

In fact, an ideal biomarker should have a series of characteristics, such as being accessible through non-invasive methods, being inexpensive to quantify, it must be specific to the disease or physiologic condition of interest and translatable from model systems to humans and, in the case of biomarkers for pathologic conditions, it should be a reliable early indicator of disease before the appearance of clinical symptoms [5]. Since miRNAs have these characteristics, they are very promising when it comes to the development of new non-invasive biomarkers [5]. However, some authors have suggested that the concentration for some miRNAs may be affected by gender and age, and due to the fact that an ideal biomarker should maintain unaltered, this information emphasizes the need of further studies, in order to uncover the miRNAs that are stable and that exhibit higher potential of being good biomarkers [11, 29].

1.2. Peripheral blood fractions

The different fractions of peripheral blood, which include plasma, serum and blood cells – leucocytes or white blood cells (WBCs), erythrocytes or red blood cells (RBCs) and platelets – differ from each other based on several factors [1]. Serum and plasma doesn't contain cells, as opposite to blood cells. Both serum and plasma contain hormones, glucose, electrolytes, antibodies, antigens and other particles. Plasma corresponds to the cell-free supernatant resulting from centrifuged blood collected in the presence of an anticoagulant [30]. On the other hand, serum is the cell-free supernatant resulting from

centrifuged blood collected in the absence of an anticoagulant, therefore, serum does not contain the most of the clotting factors, such as the fibrinogen, and cannot clot [30]. Based on these definitions, it is possible to conclude that the main difference between these two fractions relies on the presence/absence of clotting factors and associated proteins. Furthermore, during the process of coagulation, blood cells can secrete a variety of components that contribute to the different composition of plasma comparing to serum [1]. Particularly, platelets contribute with diversity of components to blood serum, such as the vascular endothelial growth factor [1].

Serum and plasma also differ in their protein content. The protein concentration in plasma is higher than in the serum [1]. The main types of proteins existent in the plasma are albumin, globulin, and fibrinogen, while the main protein components of serum consists of albumin, immunoglobulins, transferrin, haptoglobin, and lipoproteins [31, 32]. Additionally, plasma contains the Von Willebrand factor, a protein that mediates the initiation and progression of thrombus formation at sites of vascular injury, thus contributing to platelet function [33].

A study performed by Twomey and co-workers assessed the differences among serum and plasma parathyroid hormone (PTH) measurements in patients with chronic renal failure and found significant differences in the intact-PTH concentration between serum and EDTA (Ethylenediaminetetraacetic Acid) plasma in routine clinical practice [34]. This study showed that the differences between these two fractions of peripheral blood should be considered both in clinical and forensic studies [34].

Recently, it has been accepted that the fractions of peripheral blood also differ in their miRNA content.

1.3. Extracellular/circulating microRNAs

The extracellular miRNA group is diverse, considering some miRNAs can be packaged into apoptotic bodies, microvesicles (MVs), or high density lipoprotein (HDL) particles, while others are exclusively complexed with AGO proteins [3]. The vesicles group comprising both shedding vesicles and exosomes is frequently designated as MVs [25]. Apoptotic bodies are particles with a size that can range between 1 to 4μm and that remain after programmed cell death initiation [3]. Shedding vesicles are heterogeneous vesicles (100-1000 nm) that are released by outward budding and fusion of the plasma membrane, whereas exosomes are smaller vesicles (30–100 nm) that are released following the fusion of endosomal-derived multivesicular bodies with the plasma

membrane [4, 25]. MiRNAs have been identified in exosomes and microvesicles recovered from different body fluids, such as plasma, serum, saliva and urine [4, 35].

According to Arroyo and co-workers, the majority of circulating miRNAs are protein bound, rather than associated with vesicles [36]. Moreover, Turchinovich and colleagues corroborates this information, showing that extracellular miRNAs are mainly exosomes/microvesicles free and are associated with AGO proteins [37]. When plasma blood fraction is analyzed the situation is similar, as 90-95% of the totality of miRNAs in cell-debris-free blood plasma is circulating in an AGO-protein-bound form, while the microvesicle-associated miRNAs are a minority [3, 36, 37]. On the other hand, Gallo and co-workers found that the majority of miRNAs detectable in the serum of healthy individuals and systemic lupus erythematosus patients are primarily in exosomes content [35]. This divergence in the results supports the need to clarify these evidences and the need of additional studies. Moreover, although several miRNAs were detected in purified fractions of high density lipoproteins (HDL) from human plasma, their proportion in blood plasma is still unknown [3, 38]. Regarding the miRNAs packaged into apoptotic bodies, there is a lack of information concerning their contribution to the circulating miRNA population. Furthermore, in this situation, the fact that the size of apoptotic bodies is similar to that of cell debris and blood platelets and that the protocols frequently used in extracellular miRNA isolation involve the elimination of cell debris and apoptotic bodies, this may cause an overlook of the cell-free miRNA in these fractions [3].

1.4. Origin of circulating plasma and serum microRNAs

For most miRNAs present in plasma and serum their specific cellular origin is unknown, however the pattern expression of a vast number of miRNAs is established, making it possible to determine its probable source (Figure 2) [3, 25, 39].

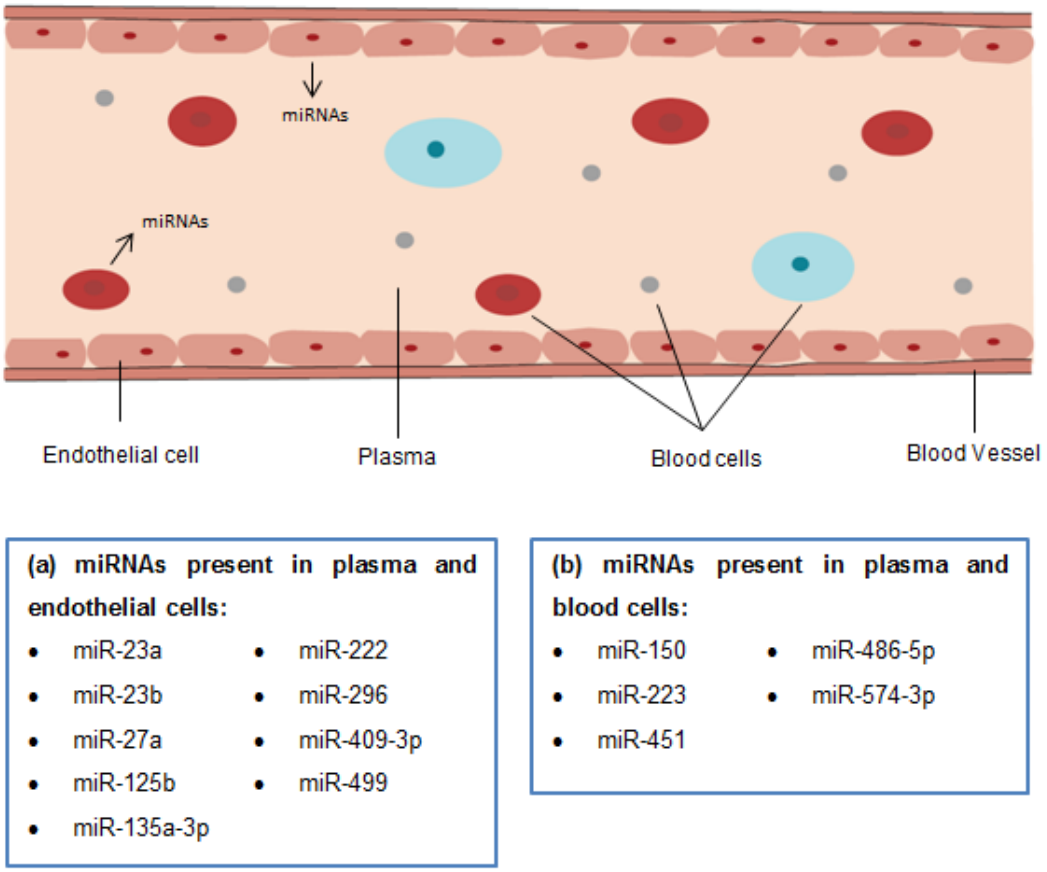

Figure 2 – Blood vessel containing plasma, blood cells (RBCs, platelets and WBCs) and endothelial cells, as well as the main miRNAs present in (a) plasma and endothelial cells and (b) plasma and blood cells. These plasma/serum miRNAs may be released by endothelial cells or/and blood cells into plasma, and thus derived from these cells.

Here, we further explore the possibility of an endothelial, blood cell and organ origin of some miRNAs present in plasma and/or serum and suggest the hypothesis that some miRNAs could originate from both endothelial and blood cells (Table 1). Since the miRNAs released from blood cells into plasma and serum are determined it would be interesting to study the alterations in the miRNA expression pattern amongst individuals with blood-related diseases in order to assess possible associations. We may consider that this can also have forensic implications, as it can be useful to the identification of the donor of a body fluid. For example, in a crime scene, in which was found a possible blood stain, but that sample is not viable for DNA extraction and analysis, since miRNAs are much more stable, this same sample might allow a miRNA analysis [4, 5]. Thus, in this particular situation, the elaboration of a miRNA expression profile might indicate specific pathologies of the sample donor, which could be essential information to narrow down the suspects list.

Table 1 – Overall of the different origins of several circulating human miRNAs.

Origin		miRNAs					References
Endothelial		miR-23a miR-222	miR-23b miR-296	miR-27a miR-409-3p	miR-125b miR-499	miR-135a-3p	[5, 7, 23, 27, 29, 40-52]
Blood cell		miR-150	miR-223	miR-451	miR-486-5p	miR-574-3p	[14, 29, 45, 46, 53, 54]
Endothelial and blood cell		let-7a	miR-16-5p	miR-22	miR-126		[5, 27, 29, 41-46, 48, 50, 53, 55-59]
	Kidneys	miR-192	miR-194	miR-204	miR-215		[5, 29, 60, 61]
	Liver	miR-122	miR-148				[3, 5, 18, 19, 29, 62-64]
Organ	**Brain**	miR-124	miR-128	miR-129-5p	miR-191	miR-342-3p	[3, 5, 19, 29, 62]
	Lungs	miR-21	miR-30b	miR-30c-1	miR-146b-5p		[5, 29, 39]
	Cancer cells	miR-7 miR-1233	miR-210	miR-221	miR-195	miR-375	[3, 20, 24, 26, 65, 66]

1.4.1. Circulating microRNAs derived from endothelial cells

The endothelium is constituted by a monolayer of cells (endothelial cells) and it is present in the internal cellular lining of the blood vessels (arteries, veins and capillaries) and the lymphatic system, thus interacting directly with the blood and lymph [67]. For this reason, it is logical to suggest that plasma and serum miRNAs that are also highly expressed in endothelial cells may derive from these cells.

For example, according to Zhou and co-workers, miRNAs encoded by the miR-23∼27∼24 gene clusters, such as miR-23a, miR-23b and miR-27a, are known to be more highly expressed in endothelial cells and in highly vascularized tissues [52]. Although these miRNAs are present in plasma, serum and blood cells, when McCall and co-workers evaluated their expression in endothelial, epithelial and hematologic cells, they found that they are more highly expressed in endothelial cells rather than in hematologic cells [29, 46]. Particularly, the expression of miR-23a and miR-23b was detected in endothelial cells by seven different profiling studies, being the miR-23a highly expressed in endothelial cells [41-44, 48-50, 57]. Similarly, miR-27a, which belongs to the miR-27 gene family, was detected in endothelial cells by five profiling studies [41, 42, 44, 49, 51].

MiR-125b and miR-409-3p can also be found in plasma and serum and although Wang and colleagues reported that these miRNAs are also present in blood cells, McCall and co-workers observed that miR-409-3p was specific of endothelial cells, presenting high expression levels in these cells and that miR-125b was expressed in endothelial and epithelial cells, and not in hematologic cells [5, 29, 46]. Moreover, the miR-125b expression in endothelial cells was confirmed by six profiling studies [41, 42, 48, 49, 51, 57].

MiR-222 can also be detected in plasma, serum and blood cells of healthy individuals, however, it is more expressed in endothelial cells, rather than in hematologic cells [29, 46]. This miRNA is involved in the endothelial cell function and seven profiling studies reported its expression in endothelial cells [27, 41, 42, 44, 45, 49-51, 57]. Furthermore, the fact that this miRNA is highly expressed in endothelial cells makes it more likely that the plasma/serum miR-222 originates from the endothelium, rather than from blood cells [43, 49]. Interestingly, a recent (and not yet published) study of our group presented evidence that when it comes to the miRNA analysis of blood cells and urine, miR-222 presents a constant expression pattern between these two body fluids [68]. Considering the expression pattern of miR-222 in endothelial cells, it would be interesting to study the same expression pattern in pathologies involving alterations in endothelial cells, such as cancer.

MiR-135a*, a miRNA that according to miRBase is now referred to as miR-135a-3p, is present in plasma and absent in blood cells [5, 7, 29]. Additionally, considering that this miRNA is expressed in endothelial cells and is not expressed in hematologic cells, the miR-135a-3p present in plasma can have an endothelial origin [46].

Another plasma miRNA that could be derived from endothelial cells is miR-296, since it is present in the plasma of healthy individuals and it has been detected in two endothelial miRNA profiling studies [41, 42, 47]. Similarly to miR-222, since miR-296 is present in endothelial cells of healthy individuals, this miRNA is also a good candidate to display expression alterations in certain types of pathologies and, therefore has potential to be studied in other types of populations.

Lastly, the miR-499, although its low expression levels in healthy individuals, this miRNA was detected in the blood plasma of healthy individuals and is highly expressed in endothelial cells, being produced almost exclusively in the heart [23, 40, 49]. The fact that this miRNA has low expression levels in the plasma of healthy individuals and is produced in the heart suggests that it might be highly expressed in individuals who exhibit cardiac diseases.

1.4.2. Circulating microRNAs derived from blood cells

The presence of miRNAs in WBCs is easily understood, as these cells have nucleus. However, when considering the platelets and RBCs the situation is different, since these cells do not have nuclei, and, therefore it is frequently thought that they do not contain nucleic acids. Chen and co-workers addressed this subject through the analysis of the transcriptome of a purified population of human mature erythrocytes from individuals with normal hemoglobin and homozygous sickle cell disease, finding that the miRNA expression in human mature erythrocytes is dramatically altered in homozygous sickle cell disease erythrocytes [69]. Additionally, their results indicated that the presence of miRNAs in RBCs may be attributed to the longer half-life and slower decay kinetics of specific miRNAs [69]. The explanation for this relies on the fact that miRNAs still exist in the functional form in reticulocytes, and that their immediate precursor, orthochromatic normoblasts, still contains nuclei [69]. Taking into consideration that the reticulocyte stage lasts for 40 hours, the reticulocyte miRNA synthesized in the orthochromatic normoblast persists in reticulocytes [69]. After the terminal differentiation, these miRNAs could persist in the mature erythrocytes [69]. Regarding the origin of platelet miRNAs, Landry *et al.* referred that pri-miRNAs are likely encoded by the genome of megakaryocytic precursor cells and converted into pre-miRNAs before platelet formation [70]. However, the fact that the mature miRNA species are much more abundant than their pre-miRNA counterparts supports the hypothesis that a large percentage of the mature miRNA content of platelets could be inherited directly from megakaryocytes [70]. Considering this information, in theory, healthy individuals should exhibit the same miRNA pattern in platelets, as opposed to individuals' carriers of diseases that affect precursor cells, whose platelets miRNA patterns should present alterations. This is another interesting line of work, since that, as referred early, the information about the pathologies of the blood sample donor might play an important role in the identification of suspects and/or victims in a forensic investigation.

MiR-223 is present in plasma, serum and blood cells – WBCs, RBCs and platelets – and although activated platelets can release miR-223 through microparticles (MPs) that can be internalized by endothelial cells, Mayr and co-workers reported that this particular miRNA is abundant in platelets and shows low expression levels in endothelial cells [29, 45, 54]. Interestingly, Pritchard *et al*. found that the plasma levels of this myeloid-enriched miRNA, closely tracked with changes in myeloid blood counts, such as platelets and neutrophils [14]. Based on this data, it is possible to conclude that the probable origin of the miR-223 is the blood cells, rather than the endothelium.

In the same way, miR-150 is present in plasma, serum, WBCs, RBCs and platelets and Pritchard *et al*. detected that the plasma levels of this lymphoid-enriched miRNA reflected lymphoid counts [14, 29].

Plasma/serum miR-451 and miR-486-5p might also originate in blood cells, since these miRNAs are both common to the cellular and the circulation miRNAs populations, being present in plasma, serum, WBCs, RBCs and platelets, being specially enriched in RBCs [14, 29, 53].

Interestingly, miR-223, miR-150, miR-451 and miR-486-5p are highly expressed in hematologic cells and are absent in endothelial cells and in epithelial cells [46].

The expression of miR-574-3p is detected in plasma, serum, WBCs, RBCs and platelets, being expressed in hematologic cells and not in endothelial cells [29, 46]. Additionally, the plasma levels of this miRNA presented significant positive correlations with myeloid blood cell counts, such as neutrophils and platelets [14].

1.4.3. Circulating microRNAs derived from endothelial and blood cells

Other circulating plasma and serum miRNAs, like miR-126, miR-22, miR-16 (currently referred to as miR-16-5p) or let-7a, which exhibit high expression levels in both blood cells as well as endothelial cells, could be equally secreted by these two types of cells into plasma/serum [5, 27, 29, 41-46, 48-50, 53, 55-59].

MiR-126, which is present in whole blood, as well as in plasma and serum, was reported to be an endothelial cell-restricted miRNA, highly expressed in the endothelium, blood vessels, heart, and lung and is involved in the endothelial cell function, suggesting an endothelial origin [27, 29, 55, 56, 58]. However, this miRNA was also identified in blood cells – WBCs, RBCs and platelets, and, although being present at lower levels in platelets that in endothelial cells, the shedding of platelet microparticles in plasma or serum also represents a major contributor to circulating miR-126 levels [29, 45].

Likewise, miR-22 is present in plasma, serum, WBCs, RBCs and platelets, being also highly expressed in endothelial cells [5, 29, 43].

MiR-16-5p is present in plasma, serum, WBCs, RBCs and platelets and it is particularly abundant in erythrocytes and endothelial cells [29, 59]. Moreover, this miRNA was detected in endothelial cells by six profiling studies [41, 42, 44, 48, 49, 57].

Let-7a, a miRNA present in plasma, serum and blood cells, common to the cellular and the circulation miRNAs populations and detected in endothelial cells by six profiling

studies is another candidate of a miRNAs derived both from endothelial and blood cells [29, 41, 42, 44, 49, 50, 53, 57].

Furthermore, McCall *et al.* observed that miR-22, miR-16-5p and let-7a are common to endothelial, epithelial and hematologic cells, and that miR-126 is expressed by endothelial and hematologic cells, supporting the hypothesis of a shared origin of these miRNAs in plasma and serum [46].

All these circulating plasma/serum miRNA that might have originated from both endothelial and blood cells could be differentiated by those which are derived by only one of these sources based on theirs expression levels in plasma and serum. It would be expected that the miRNAs that are secreted into the plasma/serum by both endothelial and blood cells would have higher levels in these fluids, when compared to the ones that are secreted either from endothelial or blood cells. As a future perspective it would be interesting to further explore this possibility in order to enlighten the miRNA origin in blood plasma and serum.

1.4.4. Circulating microRNAs with an organ origin

Due to the fact that some tissue-specific miRNAs have been identified in plasma, and when we consider the close contact between organs and the bloodstream, we can conclude that a miRNA transfer between these organs and the blood plasma/serum can occur [3]. Particularly, in highly vascularized organs, such as kidneys, liver, brain and lungs, this miRNA transfer between organs and blood plasma might represent a more significant contribution for the circulating miRNAs. For this reason, some plasma miRNAs may be organ-derived (Figure 3).

Sun *et al.* detected five miRNAs, including miR-192, miR-194, miR-204, miR-215 and miR-216, which were preferentially expressed in human kidney when compared with other organs/tissues, such as heart, spleen, lung, muscle and prostate [61]. Later, Chandrasekaran and co-workers, profiled the miRNA expression in human organs, including the kidneys, and concluded that these five miRNAs are among the few miRNAs that were found to be renal-specific [60]. Of these five miRNAs, miR-192, miR-194, miR-204 and miR-215 can be found in plasma and since they are preferentially highly expressed in the kidney, this organ might secrete them into the bloodstream, and consequently, they may have a kidney origin [5, 29, 60, 61].

Regarding normal human liver tissues, very few circulating plasma miRNAs are reported to be highly expressed in this organ. Perhaps the most well studied miRNA in

liver is miR-122, a highly expressed miRNA that represents 70% of the total miRNA pool of this organ [18]. This miRNA is also a liver-specific miRNA that can be detected in plasma, and therefore its origin in plasma might derive from the liver [3, 19, 29, 64]. Likewise, miR-148, a miRNA that can be also found in plasma presents high expression in the adult liver and the results of Barad *et al.* validated the liver-specific expression of miR-148 [5, 62, 63].

In the case of the brain, miR-124 is considered a brain-specific miRNA that have also been detected consistently in blood plasma, and therefore could be brain-derived [3, 19]. Similarly, miR-128 is present in plasma and serum and was also reported to be a brain-specific miRNA [5, 29, 62]. Other similar miRNAs are miR-129-5p, miR-191 and miR-342-3p, since that they can be found in blood plasma and exhibit high expression levels in brain in comparison with other tissues, according to miRNAMap [5, 29, 39].

In the lungs, according to miRNAMap, miR-21, miR-30b, miR-30c-1 and miR-146b-5p, are highly expressed in this organ, compared with others organs and tissues [39]. These miRNAs are also present in plasma and serum and based on theirs expression profiles, in the circulation they probably originate in the lungs, as a result of the lung irrigation process [5, 29].

Recent studies have also demonstrated a correlation between some miRNAs and specific pathologic conditions, such as cancer [3, 20, 24, 26, 65, 66]. Furthermore, since tumor cells can secrete miRNAs into the bloodstream, some studies, such as the one performed by Turchinovich *et al*., have considered the tumor as an organ that contributes to the blood miRNA population [3]. For example, the expression of serum miR-375 was reported to be greater in prostate cancer patients than in healthy individuals, and greater in metastasized cancer than in primary prostate cancer, what leads to believe that in individuals with this pathologic condition, this particularly serum miRNA could be originated in the cancer cells [26]. Moreover, this is corroborated by the study performed by Brase *et al.*, in which was found that miR-375 was highly expressed in serum of prostate cancer patients and the release of this miRNA into the bloodstream is further associated with advanced disease [20]. Another miRNA in a similar situation is miR-195. This miRNA is present in the plasma of healthy individuals and its levels were considerably elevated in samples of whole blood of patients diagnosed with breast cancer from stage I to IV, when compared to age-matched disease-free individuals [5, 24, 26]. In this study the expression of miR-195 in age- and stage-matched tumor tissues was also compared with whole blood samples and the result was that in tumor tissues and circulation, the levels of miR-195 were increased at progressive stages of breast cancer. In other words, this miRNA was more expressed in stage IV than in stage I or II [24]. This

positive correlation between tissue and circulating miR-195 suggests that this miRNA could be released into the blood by the tumor cells. MiR-221 is another example of a miRNA associated with cancer, more specifically, with the renal cell carcinoma (RCC) [66]. Teixeira *et al*. reported that patients with higher plasma levels of this miRNA presented a significantly lower survival rate, when compared to those with lower expression levels, thus, revealing miR-221 as an independent prognosis factor in RCC [66]. Furthermore, the plasma expression levels of miR-221 were higher in patients with advanced disease (metastasis at diagnosis) comparatively with patients with localized disease [66]. MiR-210 and miR-1233 are other miRNAs that are implicated in the RCC, since a recent study (and not yet published) revealed that these miRNAs presented higher expression levels in plasma samples of RCC patients, when compared to healthy individuals, being good candidates for biomarkers of prognosis and aggressiveness in RCC. The expression levels of peripheral whole-blood miR-221 and miR-7 were also reported to be implicated as potential predictive biomarkers of castration-resistant prostate cancer development [65].

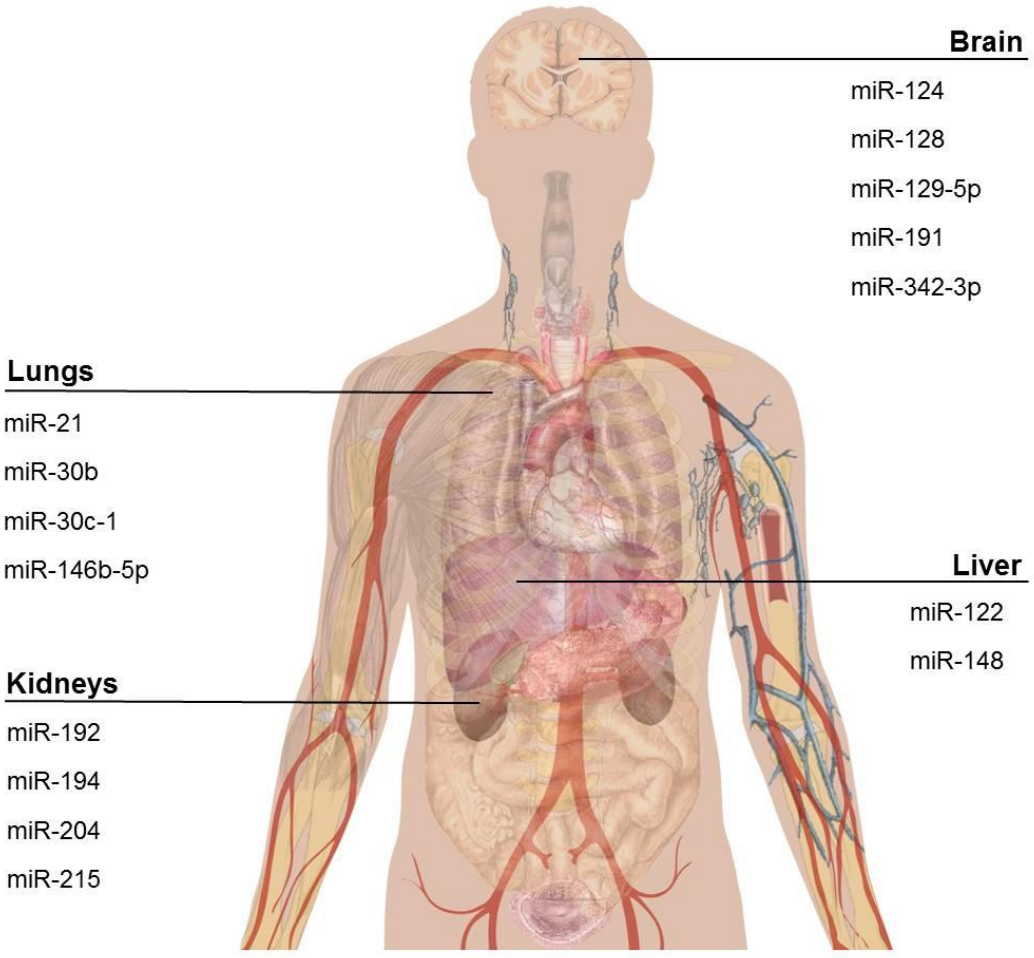

Figure 3 – Overview of several circulating plasma/serum miRNAs organ-derived. Adapted from http://upload.wikimedia.org/wikipedia/commons/6/6b/Man_shadow_with_organs.png [71]

1.5. Forensic applications of microRNAs as biomarkers for peripheral blood fractions characterization

MiRNAs biomarkers for peripheral blood fractions exhibit a wide variety of forensic applications.

One of the main aspects in the identification of miRNAs specific to peripheral blood fractions is to characterize the differences/variations existent within blood. This factor should be considered, not only in forensic sciences, but also in all the sciences that use miRNAs, since that in several studies is selected one fraction in particular without taking into account this variation. For this reason, the miRNA differences in the peripheral blood fractions should be analyzed in order to correctly select one of them according to the purpose of the study.

Another forensic application of miRNAs biomarkers for peripheral blood fractions is related to body fluid identification. In fact, during a forensic investigation the correct identification of the possible body fluids found is crucial in order to identify possible sources for DNA collection to be later used in the identification of the donor of the biologic material [13]. Within this purpose, over the years, several methods were developed towards the body fluid identification, such as serologic tests [11]. However, these methodologies exhibit low sensitivity and specificity levels [11]. Thus, due to the small size of miRNAs, theirs increased stability and the fact that they show different expression profiles according to the tissue or fluid tested, recently they have been studied, not only as a resource in the diagnostic of different pathologic conditions, but also as an alternative to the conventional methods for the forensic body fluid identification. Although miRNA profiling is not currently a standard technique in forensic investigation, with the improvement of miRNA knowledge and respective methods, it could eventually be implemented in forensic procedures. Furthermore, given the high stability of miRNAs and the fact that these potential biomarkers can be detected even in degraded samples – which are normally not legally allowed – in the future, miRNAs could lead to a change in legislation, allowing the acceptance of these types of samples as forensic evidence. This would represent a major progress and improvement in forensic investigations and evidence analysis. In the literature there are several studies about the use of miRNA biomarkers in the identification of body fluids [5, 13, 56, 72]. Courts and Madea analyzed the presence of miRNAs in blood and saliva samples of five healthy donors and presented two miRNA assays consisting of three differentially expressed miRNAs for the identification of blood (miR-126, miR-150, miR-451) and saliva (miR-200c, miR-203, miR-205) [56]. On the other hand, Hanson and co-workers evaluated the miRNA expression

profile in dried, forensically relevant biological fluids (such as blood, semen, saliva, vaginal secretions, and menstrual blood) of healthy individuals to identify possible body fluid-specific miRNAs and were able to determine a panel of nine miRNAs that were differentially expressed (miR-451, miR-16, miR-135b, miR-10b, miR-658, miR-205, miR-124a, miR-372, and miR-412), allowing the identification of the body fluid origin of forensic biological stains using only 50 pg of total RNA [13]. Other study performed by Weber and co-workers examined the presence of miRNAs in 12 human body fluids of five healthy donors, concluding that the miRNAs tested were present in all fluids analyzed and showed distinct compositions in different fluid types [5]. Zubakov and colleagues screened a set of 718 human miRNA markers in forensically relevant body fluids (saliva, venous blood, menstrual blood, semen and vaginal secretion), finding that two miRNA markers for blood (miR-144 and miR-185) and two for semen (miR-135a and miR-891a) were suggestive to be most useful for body fluid identification in forensic applications [72].

Peripheral blood fractions miRNAs profiles associated with diseases could also play a key role in forensic sciences, namely in the identification of victims and suspects or in the determination of the cause of death. For example, regarding the first case, in an investigation where there is an unidentified corpse, the determination of the best peripheral blood fraction to profile the miRNA expression, and the posterior corresponding analysis could indicate what type of diseases were present and lead to the victim identification, through the consultation of medical records. In a similar situation, in a crime scene, in which a possible blood stain was found, but a DNA comparison to blood samples from suspects can't be performed, because that stain is not viable for DNA extraction and analysis, considering that miRNAs are much more stable, this same sample might allow a miRNA analysis, which might indicate specific pathologies of the sample donor, which could be crucial information to narrow down the suspects list. Or even, in a forensic investigation, where there isn't permission for the collection of blood samples from the suspects to compare to a presumable blood stain that was found in the crime scene, in this situation, a miRNA profiling could also uncover eventual pathologies of donor of the crime scene sample. In the second case, when the cause of death is not apparent to the macroscopic/histological level or in sudden death cases, the presence of certain blood fractions miRNAs associated with diseases may indicate more clearly the factors that have caused the death.

1.6. Potential microRNA profile for peripheral blood fractions identification

Some miRNAs, due to theirs expression patterns in biological samples, have a higher potential of being specific of different fractions of peripheral blood and, therefore, could be used as biomarkers for plasma, serum, and blood cells. According to Weber and co-workers, miR-369-3p and miR-801 are unique to the plasma when compared to other body fluids such as urine, saliva, and seminal fluid [5]. Similarly, in blood cells, Wang and colleagues reported that miR-24-1* (currently referred to as miR-24-1-5p) is present in blood cells (leukocytes, erythrocytes and platelets), being absent in plasma and serum [29]. In the case of serum, it is known that miR-16 (currently referred to as miR-16-5p) is abundant in this fraction [73]. Regarding the fact of the presence of this miRNA in plasma, in the literature there is contradictory information, since Weber and co-workers didn't detected miR-16-5p in plasma and Blondal and colleagues reported that miR-16-5p is present in plasma [5, 74].

Due to the information previously described, these four miRNAs (miR-369-3p, miR-801, miR-24-1-5p and miR-16-5p) exhibit a higher potential of being exclusive of the fractions of peripheral blood, and therefore, were selected for this study.

2. Objectives

2. OBJECTIVES

2.1. Main objective

The main objective of the present study is the identification of a miRNA profile used for biological identification of the different fractions of peripheral blood, namely, plasma, serum and white blood cells.

2.2. Specific objectives

1) Systematically revision of the literature to define the miRNA profile to be evaluated in biological samples (plasma, serum and white blood cells samples);
2) Detection and determination of the expression levels of miR-801, miR-369-3p, miR-16-5p and miR-24-1-5p in plasma, serum and white blood cells peripheral blood fraction samples;
3) Specificity and sensitivity analysis regarding the miRNA profile used in the identification of the peripheral blood fractions.

3. Material & Methods

3. MATERIAL & METHODS

3.1. Study population, sample collection and processing

Peripheral venous blood samples were collected from 22 Caucasian healthy adult volunteers (17 females and 5 males) from the North of Portugal, with a mean age of 46.3 ± 17.8 years. The selected individuals didn't have any pathologic conditions, in order to prevent eventual alterations in the miRNA profiles due to certain pathologies and to assure the validity of the results. All samples were collected after all individuals signed a written informed consent to participate in the study, according to the principles of the Helsinki Declaration.

For each donor were collected two tubes, one with anticoagulant (for the collection of plasma and white blood cells), and another one without anticoagulant (for the collection of serum). The blood samples were centrifuged during 5 minutes at 2500 rpm at room temperature. After this initial centrifugation, serum and plasma were collected from the respective tubes and stored at -80ºC until molecular analysis. In order to isolate the blood cells, the whole blood samples were transferred to a 50 mL tube, to which was added 1x AKE (ammonium chloride, potassium hydrogen phosphate and EDTA). The samples were then frozen for 30 minutes at -20ºC and consequently centrifuged during 10 minutes at 2500 rpm at room temperature. The supernatant was discarded and the resulting pellet was washed using 30 mL of 1x PBS (Phosphate buffered saline) and centrifuged during 10 minutes at 2500 rpm at room temperature. The supernatant was again discarded and the pellet was diluted in 1mL of TriPure® Isolation Reagent (Roche Applied Science®).

3.2. microRNA extraction, cDNA synthesis and relative quantification

The plasma, serum and blood cell samples miRNA extraction were carried out from 350μL of the initial sample, using the GRS microRNA Kit (Grisp®), according to the manufacturer's instructions, with slight modifications in the initial steps. Briefly, for the extraction of plasma and serum miRNAs, we used 35μL of miRNA buffer and 385μL of phenol-chloroform. In the case of the WBCs samples, the lysis buffer wasn't used, as these samples were conserved in TriPure® Isolation Reagent (Roche Applied Science®), which had already caused the lysis of the cells, and 14μL of miRNA buffer, as well as 70μL of phenol-chloroform were added to the 350μL of the initial sample.

Subsequently, to evaluate the quantity and quality of RNA, 260/280nm and 260/230nm ratios and RNA concentration of each sample were determined with *NanoDrop® ND-1000.*

The cDNA synthesis (RT-PCR) was performed using the TaqMan® MicroRNA Reverse Transcription Kit according the manufacture instructions (Applied Biosystems®), wherein the extracted miRNAs were used as a template.

In order to quantify the miRNA expression levels, quantitative real-time PCR (qPCR) assays were then performed with a StepOne™ System. The reactions were performed using 1x TaqMan® Fast Advanced Master Mix (Applied Biosystems®), with 1x probes to amplify the target miRNAs (Taqman MicroRNA Assays – miR-369-3p: 000557, miR-801: 001994, miR-16-5p: 000391, miR-24-1-5p: 002440 and RNU-48: 001006) and cDNA sample. RNU-48 was used as the endogenous control. TaqMan® was selected over SYBR green and other dye-based technologies, since it is a fluorophore-tagged probe-based chemistry, and it is known to have high specificity, reproducibility and sensitivity [75]. The PCR reactions were incubated in 48-well plate at 95ºC for 20 seconds, followed by 45 cycles of 95ºC for 1 second and 60ºC for 20 seconds. A negative control was included in all reactions and for each reaction two replicas were used. The resulting data was analyzed with the StepOne Software v2.2 (Applied Biosystems®).

3.3. Statistical Analysis

The statistical analysis of the data was carried out with the computer software *IBM®SPSS®Statistics* (Version 22.0).

The $2^{-\Delta\Delta Ct}$ method (Livak method) and the Student's t test were used, in order to assess the statistical differences in the expression levels of the normalized miRNAs.

The real-time PCR assays originated Ct (Cycle threshold) values for each combination of miRNA assay-sample. These values are correspondent to the cycle number of the early exponential phase of the amplification reaction and, therefore, are inversely proportional to the relative expression levels or quantity of the miRNA in the sample tested. Thus, the mean of the Ct values within each sample type was determined and, in order to normalize the results, the difference between the mean Ct values of the target and the mean Ct values of the endogenous control (RNU-48) was assessed. This number is designated as the ΔCt ($\Delta Ct = Ct_{target} - Ct_{endogenous\ control}$).

The fold change values were calculated by the formula $2^{-\Delta\Delta Ct}$, following the equations in Figure 4.

(a) $\overline{\Delta\Delta Ct} = \overline{\Delta Ct}_{serum} - \overline{\Delta Ct}_{WBCs}$

(b) $\overline{\Delta\Delta Ct} = \overline{\Delta Ct}_{plasma} - \overline{\Delta Ct}_{WBCs}$

(c) $\overline{\Delta\Delta Ct} = \overline{\Delta Ct}_{serum} - \overline{\Delta Ct}_{plasma}$

Figure 4 – Equation for the determination of the $\overline{\Delta\Delta Ct}$ values for **(a)** serum vs WBCs; **(b)** plasma *vs*. WBCs and **(c)** serum *vs*. plasma.

The odd ratio (OR), specificity, sensitivity, positive predictive value or precision (PPV), negative predictive value (NPV), accuracy (ACC) values, as well as the receiver operating characteristic (ROC) curves were used to evaluate the potential of miR-24-1-5p as a WBCs biomarker.

4. Results & Discussion

4. RESULTS & DISCUSSION

4.1. microRNA detection frequencies

Figure 5 displays the miRNA detection frequencies of the four studied miRNAs in serum, plasma and WBCs, i.e. for each peripheral blood fraction, the graphic shows the percentage of cases in which the miRNAs were detected.

Through the analysis of this figure, we can conclude that miR-801 and miR-16-5p were both detected in the majority (95%) of the serum, plasma and WBCs samples tested. Moreover, these miRNAs have a more uniform distribution throughout the fractions, when compared to miR-369-3p and mir-24-1-5p, which were the miRNAs that exhibited more variations in the detection frequency in the several peripheral blood fractions. Overall, and based on the detection frequencies, miR-24-1-5p appears to be the miRNA with major differences in the fractions, as it was detected in 91% of the WBCs samples tested and only in 32% of the plasma samples and 5% of the serum samples tested.

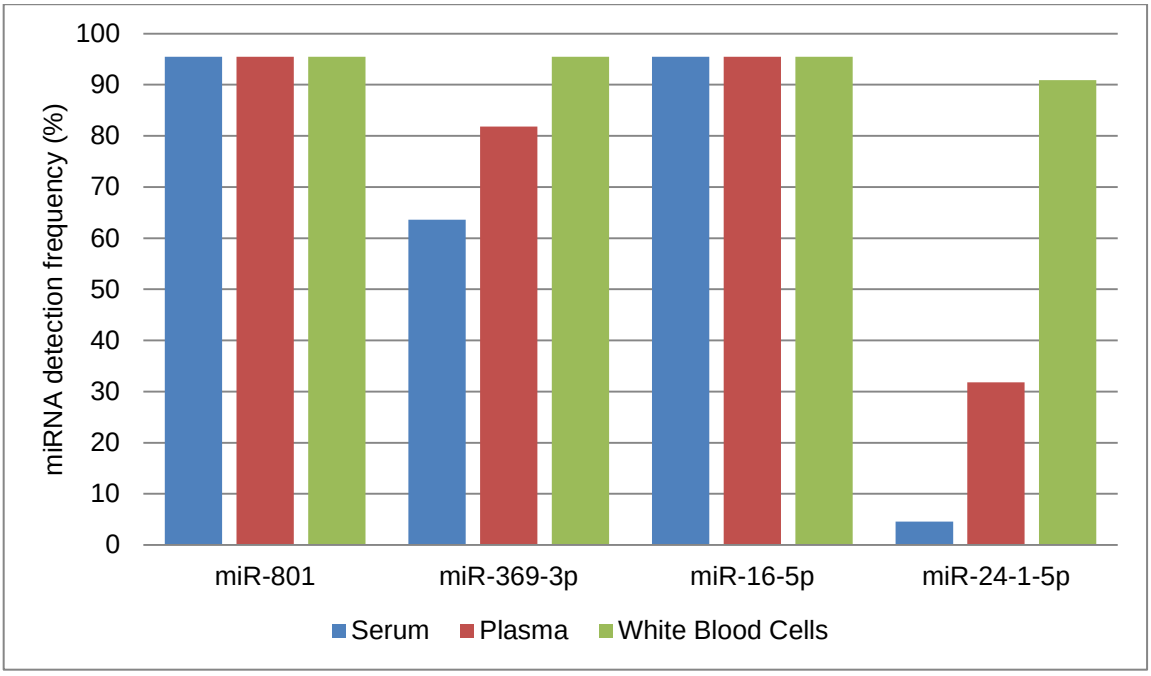

Figure 5 – miRNA detection frequencies in percentage (%) of miR-801, miR-369-3p, miR-16-5p and miR-24-1-5p in serum, plasma and white blood cells.

4.2. microRNA relative quantification in peripheral blood fractions

Figure 6 exhibits the results of the miRNA relative quantification across the four miRNAs tested (miR-801, miR-369-3p, miR-16-5p and miR-24-1-5p) in the peripheral blood fractions (serum, plasma and WBCs). The selected miRNAs show different expression patterns according to the peripheral blood fractions tested. MiR-801, miR-369-3p and miR-16-5p are expressed in all of the peripheral blood fractions, leading us to consider that these miRNAs could be good biomarkers for whole blood identification. However, these miRNAs should be tested in other body fluids in order to determine theirs effective potential as a blood biomarker. The higher expression levels of these miRNAs are observed in serum, followed by plasma and, lastly, in WBCs. This is a very interesting result, considering that in the majority of studies, plasma is preferentially used instead of serum. Based on our results, serum seems to be the optimal peripheral blood fraction for miRNA analysis, since this fraction exhibits higher miRNA content. Furthermore, within these three miRNAs, miR-16-5p is the one that exhibits higher expression levels throughout the fractions of peripheral blood, thus it might have an important role in a blood-related process. In fact, according to the literature, miR-16-5p contributes to the regulation of mammalian hematopoiesis, through the control of the terminal stages of hematopoietic development [76, 77]. Moreover, a study where the variability of miRNAs in whole blood stored until 12 hours at room temperature was assessed concluded that miR-16-5p was able to remain unaltered despite of the room temperature [78]. This stability of miR-16-5p, combined with its expression pattern in peripheral blood fractions, makes this miRNA an ideal biomarker for whole peripheral blood identification.

On the other hand, miR-24-1-5p is the miRNA that reveals a more differentiated expression pattern in the peripheral blood fractions. The expression levels of this miRNA were significantly more elevated in plasma in comparison to WBCs (P=0.001) (Figure 6). The mean -ΔCt values of miR-24-1-5p in serum are not represented in Figure 6, as this miRNA was only detected in one of the 22 serum samples tested. The fact that there were detected higher expression levels of miR-24-1-5p in plasma rather than in WBCs might appear to go against what was found through the miRNA detection frequency analysis. However, when interpreting the relative quantification results we must consider that the number of samples used to determine these values was quite different: this miRNA was found only in 7 plasma samples and the mean of the respective quantification is being compared against the relative quantification of 20 WBCs samples. We can conclude that these results related to the expression of miR-24-1-5p can represent an addition to the existent data. To the best of our knowledge, the only study to date that analyzes the

expression of this miRNA in the several fractions of peripheral blood was performed by Wang and co-workers, in which the peripheral blood fractions of 10 individuals were tested [29]. This study detected miR-24-1-5p in blood cells (leukocytes, erythrocytes and platelets), and not in plasma or serum [29]. In the present work, we took a step forward and analyzed the miRNA content of plasma, serum and WBCs from 22 individuals.

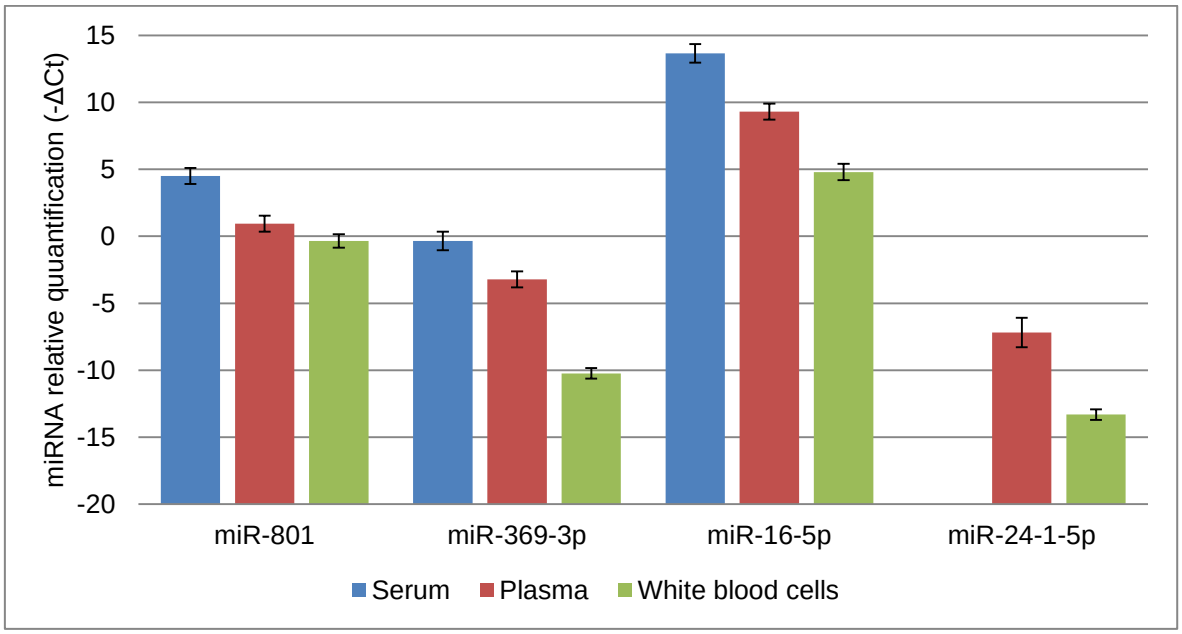

Figure 6 – miRNA relative quantification of miR-801, miR-369-3p, miR-16-5p and miR-24-1-5p in serum, plasma and white blood cells. (mean ± standard error of mean).

In Table 2, the fold change in the miRNAs expression in the different peripheral blood fractions allow us to evaluate and compare the differences between the expression levels of each of the selected miRNAs in two types of peripheral blood fractions.

Table 2 – Fold change values, and correspondent *P* values of miR-801, miR-369-3p, miR-16-5p and miR-24-1-5p in serum, plasma and white blood cells (WBCs).

	Fold change ($2^{-\Delta\Delta Ct}$) (*P* values)			
	miR-801	**miR-369-3p**	**miR-16-5p**	**miR-24-1-5p**
Serum *vs*. WBCs	28.64 (*P*<0.001)	942.27 (*P*<0.001)	461.44 (*P*<0.001)	*
Plasma *vs*. WBCs	2.45 (*P*=0.097)	129.79 (*P*<0.001)	22.63 (*P*<0.001)	70.03 (*P*=0.001)
Serum *vs*. Plasma	11.71 (*P*<0.001)	7.26 (*P*=0.109)	20.39 (*P*<0.001)	*

*Not calculated, since this miRNA was only detected in one serum sample.

The analysis of the miRNA expression levels in serum comparing to WBCs levels reveals that miR-801, miR-369-3p and miR-16-5p are, approximately, 29, 942 and 461 times, respectively more expressed in serum (P<0.001). The comparison of plasma *vs*. WBCs indicates statistically significant differences in miR-369-3p, miR-16-5p and miR-24-1-5p expression. These miRNAs are approximately 130, 23 and 70 times more expressed in plasma in comparison to WBCs (P<0.001; P<0.001 and P=0.001, respectively). As it was mentioned early, we need to approach these results with caution and in the light of the miRNA detection frequencies. Thus, for miR-24-1-5p the interpretation may not be as straightforward as one might think, as the 70 fold change value might not be a good reflection of the results, because it is based in the relative quantification of only 7 plasma samples. In order to assess whether or not miR-24-1-5p can be used as a WBCs biomarker, further statistics need to be performed (see point 4.4.). Regarding the serum *vs*. plasma fold change expression analysis, the values obtained reveal that miR-801, miR-369-3p and miR-16-5p are, respectively, 12, 7 and 20 times more expressed in serum than in plasma (P<0.001; P=0.109 and P<0.001, respectively).

The fold change analysis achieved lead us to conclude that miR-16-5p could be a good biomarker for serum identification, as its expression values allow us to distinguish this blood fraction from plasma and WBCs samples. Additionally, as it was mentioned early, this miRNA could also be a good biomarker for whole peripheral blood identification, since it showed high expression levels in all the fractions. In other words, if the screening of miR-16-5p in an unknown fluid indicates its presence, we can propose that the fluid in question is blood.

According to our results, for the miRNAs tested we observed that WBCs was the fraction that exhibit lower expression levels (Figure 6, Table 2). This is an interesting result, especially when taking into account that miRNAs are produced in cells and, hence, without any additional knowledge, it would be expected to find higher expression levels in WBCs. This illustrates the fact that miRNAs, despite originating in cells, can and do, indeed, exhibit different expression patterns amongst the fluid or tissue tested, due to the fact that cells are able to export miRNAs into the extracellular environment [3]. In this particular case, we observed higher expression levels in serum, which means that, for these miRNAs, there is a larger quantity of miRNA molecules in serum, in comparison to WBCs and plasma. For miR-24-1-5p, as stated early, the interpretation of the results isn't as straightforward as it is for the other miRNAs, and, with these results we cannot yet dismiss this miRNA as a WBCs biomarker.

4.3. microRNA profile according to age and gender

In this section, we analyzed the relative expression of the miRNAs tested (miR-801, miR-369-3p, miR-16-5p and miR-24-1-5p) in the peripheral blood fractions (serum, plasma and WBCs) according to the age and gender.

Since our study population had a median age of 48.5, we divided the results in two different groups (< 48 years and ≥ 48 years).

As shown in Figure 7, the majority of miRNAs showed no statistically significant differences according to the age in the three samples tested (serum, plasma and WBCs). The only case that exhibited significant differences was miR-369-3p in serum and plasma. According to the results, the expression levels of miR-369-3p were significantly higher in young individuals (age < 48 years) than in the older group (age ≥ 48 years) in serum (*P*=0.027) and in plasma samples (*P*=0.023).

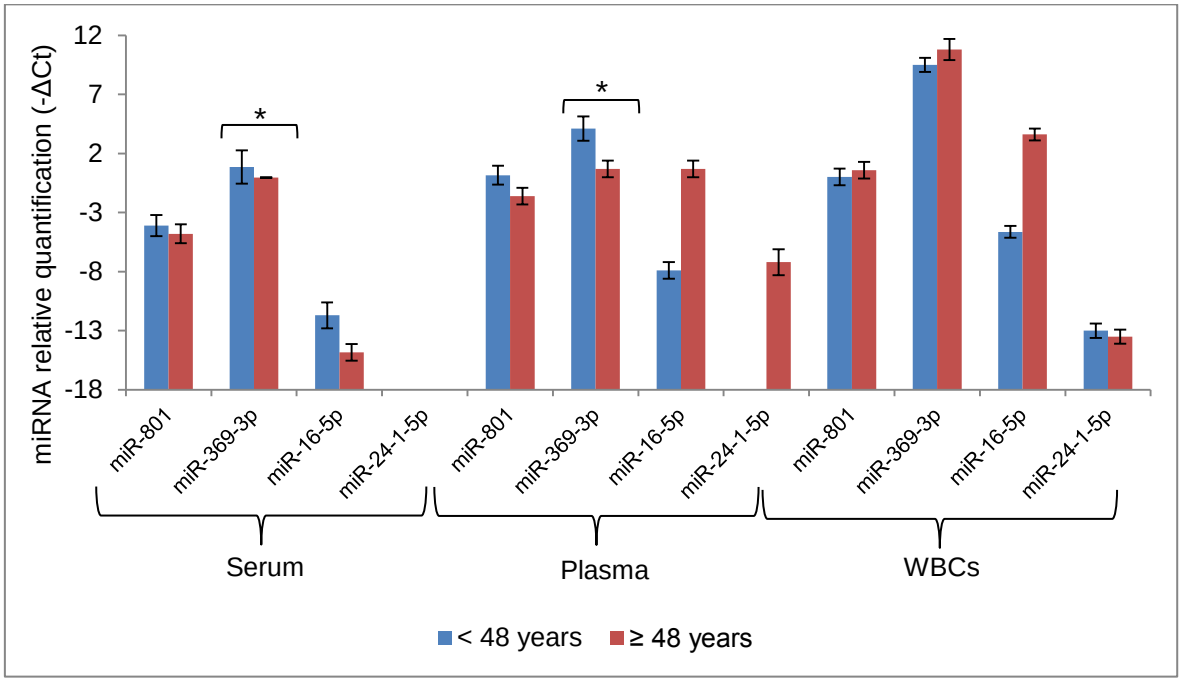

Figure 7 – miRNA relative quantification of miR-801, miR-369-3p, miR-16-5p and miR-24-1-5p in serum, plasma and white blood cells (WBCs) according to age (mean ± standard error of the mean; * *P* < 0.050).

In the analysis according to gender, the situation is similar to what it was observed for age, since predominantly weren't found any statistically significant differences according to this characteristic, with the exception of miR-16-5p in serum (*P*=0.027) and miR-801 in WBCs (*P*=0.025), which were the only cases that exhibited significant differences, i.e., the expression levels of miR-801 in WBCs and of miR-16-5p in serum were significant higher in males, than in females (Figure 8).

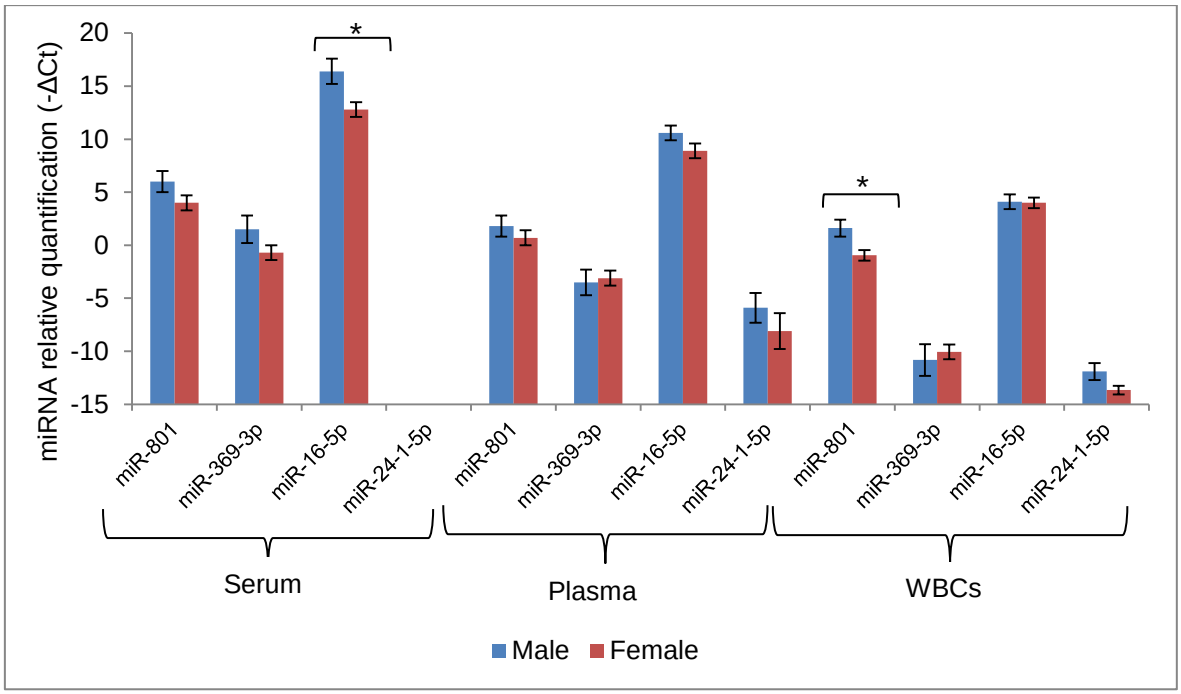

Figure 8 – miRNA relative quantification of miR-801, miR-369-3p, miR-16-5p and miR-24-1-5p in serum, plasma and white blood cells (WBCs) according to gender. (mean ± standard error of the mean; * $P < 0.050$).

4.4. Specificity and sensitivity analysis of miR-24-1-5p as a WBCs biomarker

In order to clarify and evaluate the effective potential of miR-24-1-5p as a WBCs biomarker, a ROC curve was constructed and odd ratio (OR), specificity (or true negative rate, TNR), sensitivity (or true positive rate, TPR), positive predictive value or precision (PPV), negative predictive value (NPV) and accuracy (ACC) values were determined (Table 3, Figure 9). These statistics were performed since they are able to reflect the ability of the correct identification of a peripheral blood fraction as WBCs or non-WBCs (serum and plasma) based on the detection or non-detection of this miRNA in the sample in question [79, 80].

The odd ratio (OR) represents the odds of the sample being WBCs, given that miR-24-1-5p is present, compared to the odds of the sample being plasma or serum in the presence of miR-24-1-5p [81]. The specificity reveals the proportion of the non-detection of miR-24-1-5p in a sample that isn't WBCs (serum and plasma) [80]. On the other hand, the sensitivity reflects the proportion of the detection of miR-24-1-5p in WBCs samples [80]. The positive predictive value (PPV) is the proportion of WBCs samples identified in which miR-24-1-5p was detected, whereas the negative predictive value (NPV) indicates the proportion of serum and plasma in the samples in which miR-24-1-5p wasn't detected [80]. Lastly, the accuracy (ACC) is the proportion of correctly identified fractions [80].

According to our results, we observed that the presence of miR-24-1-5p in biological samples presents a likelihood of 45 times higher to be WBCs than serum or plasma (OR=45) (Table 3, Figure 9). Moreover, the specificity of miR-24-1-5p is 81.82%, and the sensitivity is 90.91%. Additionally, the PPV value is 71.43%, and the NPV value is 94.74%. The ACC is 84.85%, in other words, with the screening of miR-24-1-5p in an unknown fraction, 84.85% of the samples tested are correctly identified as WBCs or non-WBCs (serum and plasma). These results suggest that miR-24-1-5p is a potential biomarker for WBCs identification.

Table 3 – Odd Ratio (OR), specificity, sensitivity, positive predictive value or precision (PPV), negative predictive value (NPV) and accuracy (ACC) values for the evaluation of miR-24-1-5p as a WBCs biomarker.

		OR	Specificity (%)	Sensitivity (%)	PPV (%)	NPV (%)	ACC (%)
All samples		45	81.82	90.91	71.43	94.74	84.85
Age	**< 48 years**	10	94.44	100.00	90.00	100.00	96.30
	≥ 48 years	14	73.08	84.62	61.11	90.48	76.92
Gender	**Female**	92	85.29	94.12	76.19	96.97	88.00
	Male	92	70.00	80.00	57.14	87.50	73.33

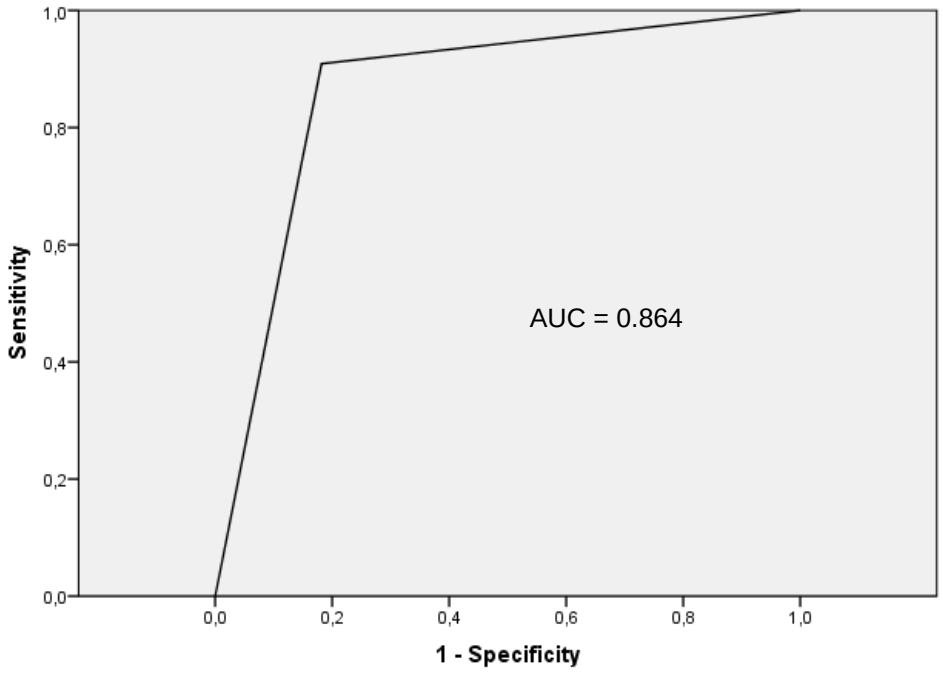

Figure 9 – ROC curve of miR-24-1-5p as a WBCs biomarker. AUC – Area under the curve.

Furthermore, we analyzed the efficiency of miR-24-1-5p in the identification of WBCs according to age and gender (Table 3). We observed that the reliability of this miRNA remained elevated across all the groups tested, exhibiting only a small reduction in the statistic values in the male group, comparing to the female and in the ≥ 48 years age range, in comparison to the < 48 years age group. Thus, this miRNA appears to be more effective in younger individuals and in females. A factor that might have impacted the lower values within the male group is the number of individuals, which were only 5, while the female group consisted of 17 individuals. Moreover, we observed that the miR-24-1-5p ROC curve has an area under the curve (AUC) of 0.864 (Figure 9). The AUC ranges between 0 and 1, wherein higher values indicates a superior test performance [80, 82]. Thus, in this particular situation, the AUC value of 0.864 indicates that the miR-24-1-5p screening has a probability of 86.4% to correctly identify two samples, one of which is WBCs and the other is non-WBCs (serum and plasma). Based on these results, we can state that miR-24-1-5p can be a biomarker for WBCs identification.

In order to better understand the significance of our results regarding the WBCs identification, we compared the specificity, sensitivity and AUC values of miR-24-1-5p to the other tested miRNAs (miR-801, miR-369-3p and miR-16-5p) (Table 4). These results demonstrate that miR-24-1-5p has the higher specificity and AUC values and is, in fact, the miRNA with the better differentiation potential between WBCs and the other examined fractions (serum and plasma).

Table 4 – Comparison of the specificity, sensitivity and area under the curve (AUC) values for the identification of WBCs, of miR-24-1-5p with miR-369-3p, miR-801 and miR-16-5p.

miRNAs	Specificity (%)	Sensitivity (%)	AUC
miR-24-1-5p	81.8	90.9	0.864
miR-801	6.8	95.5	0.511
miR-369-3p	25.6	100.0	0.628
miR-16-5p	4.5	95.5	0.500

We also performed a comparison of the specificity and sensitivity values of miR-24-1-5p in our study with previously establish forensic and clinic biomarkers/tests that are currently used or that its use has been suggested (Table 5). For example, Ethyl Glucuronide, an ethanol metabolite used to test ethanol consumption in forensic scenarios, when tested in urine can discriminate between nondrinkers and individuals sober more than 4 days *vs*. individuals that drank in the recent 4 days with a sensitivity of 83.50% and a specificity of 68.30% [83]. Other example is the initial arterial lactate level, a parameter that can be detected in blood, which was been identified as potential prognostic value of acute paraquat (herbicide widely used in agriculture) poisoning and has a specificity of 79.20% and a sensitivity of 84.60% [84]. We can additionally mention the Papanicolaou test, which is a well-known and frequently used test in clinical practice for cervical-cancer screening [85]. The specificity of this test for cervical intraepithelial neoplasia of grade 2 or 3 identification is 96.80%, whereas the sensitivity is 55.40% [85].

Based on our results we can verify that miR-24-1-5p, not only displays elevated levels of OR, specificity, sensitivity, PPV, NPV, ACC, and AUC but also, exhibits higher specificity and sensitivity levels than some other tests mentioned in the literature, and, therefore, has a potential to be a remarkable biomarker for WBCs identification.

Table 5 – Comparison of specificity and sensitivity values of miR-24-1-5p for WBCs identification and currently/potential used biomarkers/tests, such as the Ethyl Glucuronide (for ethanol consumption identification), the initial arterial lactate level (for acute paraquat poisoning) and the Papanicolaou test (for cervical-cancer screening).

Biomarkers	Specificity (%)	Sensitivity (%)	References
miR-24-1-5p	**81.82**	**90.91**	**Current work**
Ethyl Glucuronide	68.30	83.50	[83]
Initial arterial lactate level	79.20	84.60	[84]
Papanicolaou test	96.80	55.40	[85]

5. Conclusion & Future Perspectives

5. CONCLUSION & FUTURE PERSPECTIVES

The use of miRNAs as biomarkers for body fluid and pathologies identification as an auxiliary method in the forensic investigation, notwithstanding being a relatively recent field, it is a very promising area. Furthermore, as it was mentioned throughout this work, peripheral blood miRNAs are very promising biomarkers in forensic investigation, having several applications. In the case of body fluid identification, the detection of the blood-specific miRNAs (whether these being whole blood, plasma, serum or blood cell specific miRNA) might be very useful to the identification of the donor of the sample, as it helps, for instance, in the identification of possible DNA collection sources. Another application, as stated early, is the fact that miRNAs with a differentiated expression pattern between heathy individuals and individuals with certain pathologies can be very useful in the identification of suspects and/or victims in a forensic investigation and, therefore, might help to reduce the list of suspects. Additionally, since miRNAs have a high stability and can be detected even in degraded samples, with the constant increasing knowledge in this area, eventually, they could allow the legal acceptance of this type of samples, and, consequently, could revolutionize the way evidences are processed, analyzed and used in court.

However, despite all the advantages of the use of miRNAs as biomarkers, additional studies are necessary to overcome the information gaps existent, for example, in respect to the miRNA origin in the fractions of peripheral blood, as well as the mechanism in which these miRNAs reach the bloodstream.

Our results demonstrated that all of the miRNAs tested were found in, at least one sample of each peripheral blood fraction, however, we were able to detect different expression patterns. The main conclusions reached are the following: (1) the OR, specificity, sensitivity, PPV, NPV, ACC and AUC values of miR-24-1-5p in WBCs identification reveal that this miRNA can be a biomarker for WBCs identification; (2) when compared to the other fractions, as serum exhibited the higher miRNA expression levels for miR-801, miR-369-3p and miR-16-5p, this fluid represents the best option for miRNA analysis; (3) since miR-801, miR-369-3p and miR-16-5p were expressed throughout all the blood fractions tested, these miRNAs could be biomarkers for whole blood identification, however, in order to assess theirs effective potential as a blood biomarkers it would be essential to test these three miRNAs in other body fluids; (4) miR-16-5p could also be a biomarker for serum, however it should be tested in a larger set of samples, in order to allow the clarification of its potential and its use as a serum biomarker should be

performed with caution, as it quantification exhibited statistically significant differences according to gender (Table 6).

Table 6 – Main conclusions reached concerning the biomarker potential of the analyzed miRNAs.

miRNAs	Biomarker	
miR-24-1-5p	WBCs	• Detected almost exclusively in WBCs • Sensitivity of 90.91% and specificity of 81.82% for WBCs identification
miR-16-5p	Serum	• Overly expressed in serum, comparing to plasma and WBCs (statistically significant)
	Peripheral blood	• Expressed in all peripheral blood fractions
miR-801 and miR-369-3p	Peripheral blood	• Expressed in all peripheral blood fractions

Furthermore, the fact that, overall, the miRNA expression pattern of the analyzed miRNAs didn't reveal any major differences between samples from individuals of different gender or age, in a forensic perspective, represents a solid result, since that, in forensic body fluid identification what is desirable is a miRNA that could allow the correct identification of the fluid/fraction throughout the population, without displaying individual variations.

Overall, our results demonstrated that miR-801, miR-369-3p and miR-16-5p could be biomarkers for whole blood identification; miR-16-5p could additionally be a biomarker for serum identification and miR-24-1-5p for WBCs identification.

In the future it would be useful to work towards the standardization of the procedures for miRNA analysis, in order to allow a more reliable interpretation of the results between studies. Moreover, as future perspectives it would be also interesting to explore the expression patterns and levels of these and others miRNAs in peripheral blood fractions in a larger and diverse set of samples, to uncover other miRNAs that could be used as biomarkers for these fractions, as well as possible associations with pathologic conditions. Particularly, it would be interesting to pursue this line of investigation for miR-24-1-5p. Since the present work uncover that this miRNA is a good biomarker for WBCs, the search for potential pathologic conditions that could be identified through the detection of alterations in its normal expression levels, could be a major tool in forensic

investigations, for example, in the determination of the cause of dead. Thus, the improvement and increase of knowledge in this area, could lead to the widespread use of miRNAs in forensic labs and, consequently, these molecules could one day play a key role in forensic investigations.

6. References

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7. Attachments

7. ATTACHMENTS

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Life Sciences

microRNAs for peripheral blood fractions identification: origin, pathways and forensic relevance

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs, with a length of 18 to 24 nucleotides that play a regulatory role in several cellular processes. Since their discovery, they have been identified in cells, tissues, organs, and body fluids and their potential as molecular biomarkers for the diagnosis of various pathologic conditions has been explored. However, little is known about the origin of the extracellular miRNAs and what factors influence the levels of circulating miRNAs. This information could help the refinement of miRNAs as more effective biomarkers. Additionally, the identification of the origin of miRNAs may prove to be very useful in the association of particular miRNAs with specific pathologies.

This review aims to gather information concerning the origin of miRNAs in plasma and serum, as well as to assess their potential to be use as biomarkers for these peripheral blood fractions.